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Perinuclear localization of the HIV-1 regulatory protein Vpr is important for induction of G2-arrest

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ABSTRACT

The HIV-1 accessory protein Vpr induces G2 cell cycle arrest and apoptosis. Previous studies indicate that the induction of G2-arrest requires the localization of Vpr to the nuclear envelope. Here we show that treatment of Vpr-expressing HeLa cells with the caspase 3 inhibitor Z-DEVD-fmk induced accumulation of Vpr at the nuclear lamina, while other proteins or structures of the nuclear envelope were not influenced. Furthermore, Z-DEVD-fmk enhances the Vpr-mediated G2-arrest that even occurred in HIV-1_{NL4-3}-infected T-cells. Mutation of Pro-35, which is important for the integrity of helix-α1 in Vpr, completely abrogated the Z-DEVD-fmk-mediated accumulation of Vpr at the nuclear lamina and the enhancement of G2-arrest. As expected, inhibition of caspase 3 reduced the induction of apoptosis by Vpr. Taken together, we could show that besides its role in Vpr-mediated apoptosis induction caspase 3 influences the localization of Vpr at the nuclear envelope and thereby augments the Vpr-induced G2-arrest.

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Findings

Background

The 96 amino acid Vpr is a virion-associated (Paxton et al., 1993), nucleo-cytoplasmic shuttling protein (Sherman et al., 2001) that is highly conserved among primate lentiviruses. Vpr is reported to exhibit numerous biological activities including nuclear localization (Sherman et al., 2001), induction of apoptosis (Roshal et al., 2001), and protein transduction through cell membranes (Henklein et al., 2000). Another well-established biological function of Vpr is its ability to cause HIV-1-infected proliferating CD4$^+$ T-cells to undergo an arrest or delay at the G2 cell cycle checkpoint (Jowett et al., 1995). A potential explanation for the obvious paradigm that Vpr prevents proliferation of infected T-cells by arresting them in the G2-phase was provided by the observation that viral gene expression is optimal in the G2-phase and that Vpr can increase virus production by delaying cells at this stage of the cell cycle (Goh et al., 1998). However, the function of the G2-arrest for HIV-1 replication is still not fully understood.

In an earlier report it was suggested that localization of Vpr at the nuclear envelope (NE) is strictly required for its capability to induce G2 cell cycle arrest (Di Marzio et al., 1995; Jacquot et al., 2007). Point mutations within the helix α-1 of Vpr (L23F, K27M) disrupted the binding to the nuclear lamina and thus abrogated Vpr-mediated G2-arrest (Jacquot et al., 2007). Another important function of Vpr is the induction of apoptosis in HIV-1 infected cells *via* destabilization of the mitochondrial membrane leading to cytochrome C release and activation of the caspase 9/3 pathway (Andersen et al., 2008; Votteler et al., 2007). During induction of apoptosis, caspase 3 is translocated from the cytoplasm into the nucleus *via* active transport through nuclear pores (Kuwana et al., 1998; Yasuhara et al., 1997). Generally, the induction of apoptosis and activation of the caspase 9/3 cascade causes changes in the diffusion limit of nuclear pores (Buendia et al., 1999; Faleiro and Lazebnik, 2000). Considering the observation that both caspase 3 and Vpr, were shown to influence the diffusion limit of the nuclear pore complex (Faleiro and Lazebnik,

Abbreviations: CD4, cluster of differentiation 4; Cul4A, Cullin 4°; DAPI, 4′, 6-diamidino-2-phenylindole; DCAF1, DDB1-cullin 4-associated-factor; DDB1, DNA-damage-binding protein 1; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus 1; MDM, monocyte derived macrophage; NE, nuclear envelope; PBMC, peripheral blood mononuclear cell; PFA, paraformaldehyde; Pro, proline; SD, standard deviation; Vpr, viral protein R; VprBP, viral protein R binding protein; WST1, water soluble tetrazolium

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2000; Sherman and Greene, 2002), and the fact that localization of Vpr at the NE is important for induction of G2-arrest (Fouchier et al., 1998; Jacquot et al., 2007; Le Rouzic and Benichou, 2005; Popov et al., 1998), we set out to investigate whether caspase 3-inhibition affects sub-cellular localization and activities of Vpr in apoptosis and G2-arrest induction.

Results

Caspase 3-inhibition causes accumulation of Vpr at the NE

In order to investigate the impact of caspase 3-inhibition on localization and function of Vpr the cell permeable, specific caspase 3 inhibitor Z-DEVD-fmk was used. This compound irreversibly inhibits the activation of caspase 3 (Garcia-Calvo et al., 1998). To analyze the sub-cellular localization of Vpr, HeLa cells were transiently transfected with plasmids expressing N-terminally FLAG-tagged Vpr (*wt,* L23F, P35A) (Votteler et al., 2007) and treated with 40 μM Z-DEVD-fmk for 24 h. Cells were then fixed, permeabilized, stained with a FLAG-specific, FITC-conjugated antibody and subjected to confocal laser scanning microscopy. Structures of the nucleus were stained with DAPI. Normal subcellular distribution of *wt* Vpr was observed in the nucleus and the cytoplasm as described earlier (Fig. 1A) (Le Rouzic and Benichou, 2005). In contrast to previous reports (Jacquot et al., 2007), in our experimental set-up it was not possible to detect a fraction of *wt* Vpr at the NE. This might be explained by the different protocols used for microscopy and was also observed by others (Belzile et al., 2010). However, and most intriguingly, treatment of cells with Z-DEVD-fmk for 24 h induced a strong perinuclear rim staining of Vpr that localized at the outer lines of the nuclear DAPI staining. This most likely represents the accumulation of Vpr at the NE that parallels with reduced levels of Vpr in the nucleus (Fig. 1B). This phenomenon not only occurred in single cells, but was the predominant phenotype for cells treated with Z-DEVD-fmk (Fig. 2A).

As shown previously (Jacquot et al., 2007), the VprL23F mutant was no longer able to bind to the NE (Fig. 1E) and, in contrast to *wt* Vpr, showed no accumulation at the NE upon Z-DEVD-fmk treatment, indicating that this accumulation of Vpr at the NE depends on a specific interaction between Vpr and components of the NE (Fig. 1F). It was suggested that the N-terminal helix α-1 somehow mediates the binding of Vpr to the NE (Jacquot et al., 2007). Thus, we further tested the influence of helix α-1 using our previously characterized VprP35A mutant (Votteler et al., 2007). In this mutant helix α-1, which is delineated by the C-terminal Pro-35 in *wt* Vpr, is extended towards the C-terminus resulting in a reduced half life and virion incorporation of the protein (Votteler et al., 2007). Though, in contrast to the VprL23F mutant, the VprP35A mutant contains a *wt* sequence of the helix α-1 (Votteler et al., 2007) and induces G2 cell cycle arrest at the level of the *wt* protein (Fig. 4C) (Ardon et al., 2006; Votteler et al., 2007). However, mutation of Pro-35 to alanine renders the mutant insensitive to caspase 3-inhibition and abrogates the accumulation of Vpr at the NE (Fig. 1D), indicating that the effect of caspase 3-inhibition is specific for *wt* Vpr.

In order to exclude an unspecific effect of Z-DEVD-fmk on the structure and integrity of the nuclear lamina and the localization of the nuclear pores, HeLa cells were stained with antibodies specific for the nuclear lamina and the nuclear pore complex. The structure of the nuclear lamina and the localization of the nuclear pore complex remained unchanged upon Z-DEVD-fmk treatment (Fig. 2B and C; first column).

To examine the localization of another protein that binds to the NE, HeLa cells were transfected with the nuclear pore localizing protein RanGAP which was fused to eGFP (Joseph et al., 2002) and cells were treated with Z-DEVD-fmk. Besides its cytoplasmatic localization, RanGAP co-localizes at the nuclear pore complex (Fig. 2C) (Matunis et al., 1996). However, and in contrast to Vpr, inhibition of caspase 3 did not change the localization of this NE-associated protein (Fig. 2B and C), indicating that the Z-DEVD-fmk effect is specific for Vpr.

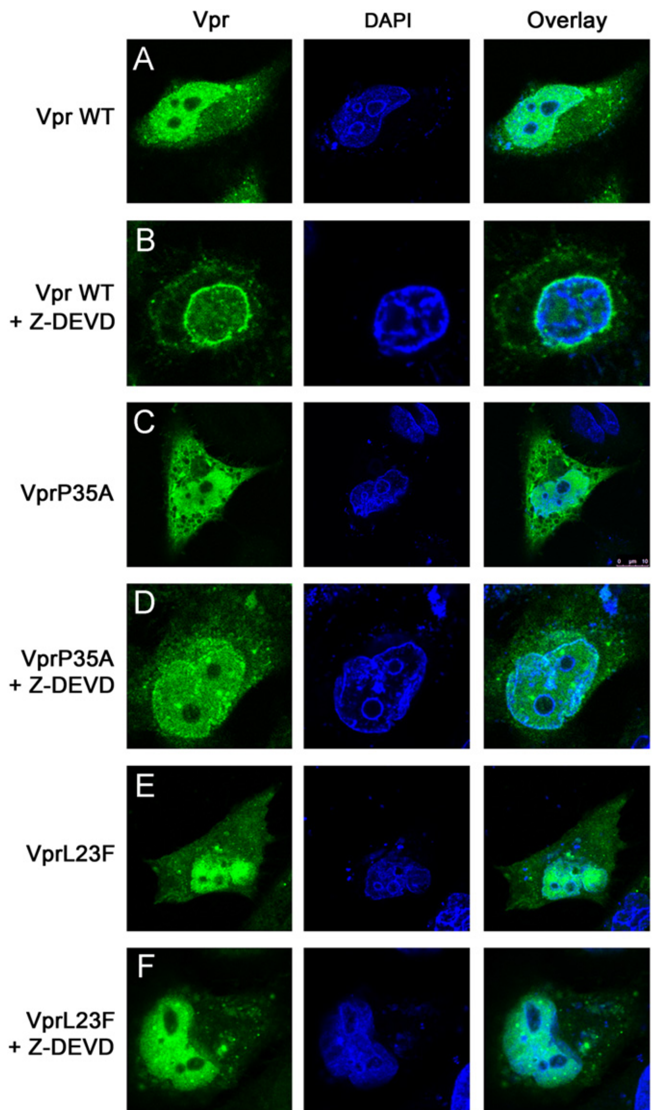

Fig. 1. Sub-cellular localization of Vpr: HeLa cells were transiently transfected with pCMV-FLAG-Vpr constructs. FLAG-Vpr was stained with a FLAG-specific, FITC-conjugated antibody (green signal). Nuclear compartments were counterstained with DAPI (blue signal). Cells in (B), (D), (F) were treated with Z-DEVD-fmk. Controls in (A), (C), (E) were treated with DMSO for solvent control.

Z-DEVD-fmk treatment affects neither the half life and the virion incorporation of Vpr nor its contribution to virus replication

Having shown that treatment of cells with Z-DEVD-fmk enhances the perinuclear localization of Vpr without changing the overall structure of the NE, we next wanted to analyze whether caspase 3-inhibition influences the stability and virus-incorporation of Vpr. Although the localization of *wt* Vpr was affected by Z-DEVD-fmk, its half life (Fig. 3A) and virion incorporation (Fig. 3B) is not dependent on caspase 3-activity. The mutant VprP35A, which does not change its localization upon Z-DEVD-fmk treatment (Fig. 1) was also not affected by caspase

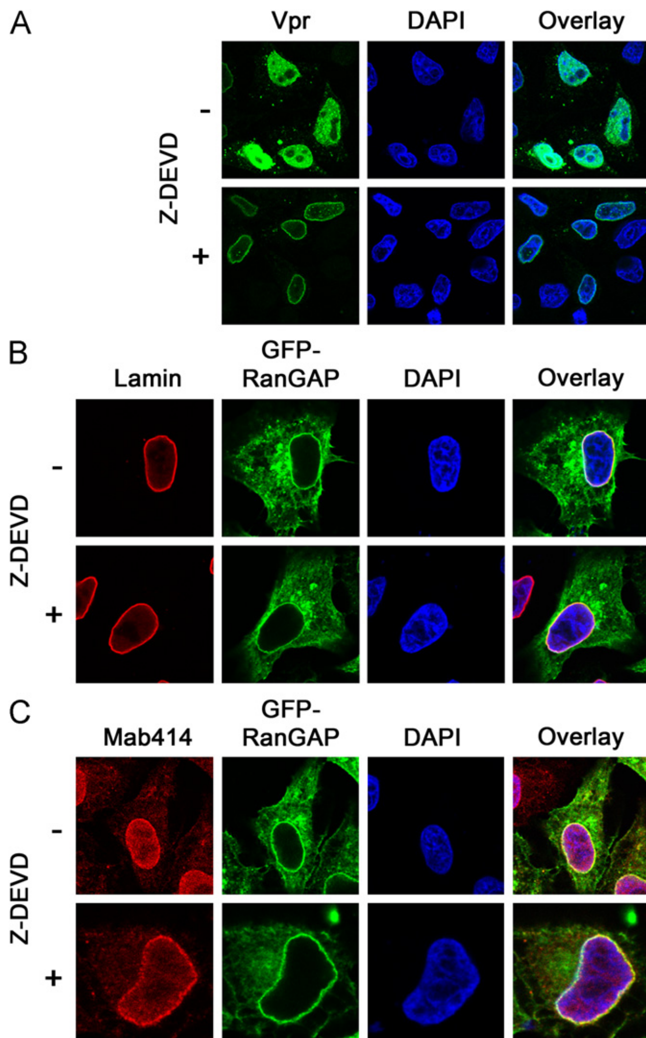

Fig. 2. Sub-cellular localization of Vpr: (A) Greater section of HeLa cells with or without Z-DEVD-fmk treatment. (B), (C) Localization of RanGAP after caspase 3-inhibition. HeLa cells were transfected with peGFP-RanGAP (Joseph et al., 2002), treated with Z-DEVD-fmk for 24 h and stained with (B) Lamin A/C antibody (red signal), specific for the nuclear lamina, or (C) Mab414 antibody (red signal), specific for the nuclear pore complex. Nuclear compartments were counterstained with DAPI (blue signal). Green signal: eGFP-RanGAP.

3-inhibition in terms of half life and virion incorporation (Fig. 3A and B). As shown earlier, due to the structural changes induced by the P35A mutation, the VprP35A protein is not efficiently incorporated into budding virions (Fig. 3B) (Votteler et al., 2007).

To exclude an unspecific or cytotoxic effect of Z-DEVD-fmk, cell viability was tested in HeLa cells using a WST-1 assay, demonstrating that the concentration of 40 μM Z-DEVD-fmk showed no adverse effects (Fig. 3C).

As induction of G2-arrest was postulated to enhance replication of HIV-1 (Goh et al., 1998), we finally wanted to test whether caspase 3-inhibition somehow affects Vpr-dependent replication of HIV-1. Because accessory proteins like Vpr particularly deploy their function in primary cells, monocyte derived macrophages (MDM) and human PBMCs were infected with HIV-1_{NL4-3}-derived X4- or R5 tropic viruses and treated with Z-DEVD-fmk. However, virus replication was not affected by caspase 3-inhibition, irrespectively of the co-receptor tropism (Fig. 3D). Studies in PBMCs and MDMs were repeated at least three times with the same experimental outcome. Of note, replication studies were also performed in permanent T-cell culture (Jurkat), exhibiting the same result as observed in primary human cells (data not shown). Nevertheless, we cannot fully exclude a possible influence of caspase 3-inhibition on HIV-1 replication as a result of augmented Vpr-induced G2 cell cycle arrest.

Induction of G2 cell cycle arrest by Vpr is increased under caspase 3-inhibition

Considering that localization of Vpr at the NE is important for induction of Vpr-mediated G2-arrest (Jacquot et al., 2007), it was legitimate to ask whether or not the increased accumulation of Vpr at the NE after Z-DEVD-fmk treatment would affect this major function of Vpr. We also included the mutant VprP35A, as it is capable of inducing G2-arrest (Ardon et al., 2006; Votteler et al., 2007) while it is insensitive to Z-DEVD-fmk treatment in terms of localization at the NE (Fig. 1).

To investigate the Vpr-induced cell cycle arrest, Jurkat T-cells were infected with either *wt* HIV-1_{NL4-3}, the VprP35A mutant thereof, or a *vpr*-deletion-variant (HIV-$1_{NL4-3}\Delta vpr$). Cells were treated with Z-DEVD-fmk starting at 2 h post infection and medium as well as inhibitor were replaced every 3rd day. At peak of infection, cells were stained with a FITC-labelled, monoclonal anti-p24 antibody to gate for HIV-1-infected cells by flow cytometry. The cell cycle state was quantified by DNA staining. As shown in Fig. 4B, the G2-arrest induction observed for *wt* HIV-1_{NL4-3}-infected cells was considerably elevated during caspase 3-inhibition compared to the untreated control (Fig. 4A). Cells infected with HIV-$1_{NL4-3}\Delta vpr$ showed an overall reduced G2-arrest level (Fig. 4E), which was not enhanced upon Z-DEVD-fmk treatment (Fig. 4F). This indicates that the enhanced G2-arrest under caspase 3-inhibition is specific for Vpr and does not result from unspecific effects of the inhibitor.

Consistent with previous reports (Ardon et al., 2006; Votteler et al., 2007), and in contrast to the mutant VprL23F (Jacquot et al., 2007) the HIV-1_{NL4-3}VprP35A mutant still induced G2-arrest at levels comparable to *wt* Vpr (Fig. 4C). However, caspase 3-inhibition did not increase the level of G2-arrest induced by VprP35A. This further confirms the notion that NE localization of Vpr is closely linked to G2-arrest induction, as localization of VprP35A was shown to be insensitive to Z-DEVD-fmk (Fig. 4D). This effect might be consequent to the fact that the P35A mutation did not destroy but rather extend helix α-1 in Vpr (Votteler et al., 2007). One possible explanation for the phenomenon could also be that the inhibition of apoptosis would be the underlying reason for the augmentation of G2 arrest upon caspase 3-inhibition since more cells would survive in an acutely infected stage. However, if this was the case, the mutant VprP35A, which does not respond to Z-DEVD treatment in terms of G2-arrest, yet is active in terms of apoptosis as wt Vpr (Votteler et al., 2007), would show the same augmentation in G2-arrest induction upon caspase 3 inhibition as the *wt* protein. According to three independent studies, the mean increase of *wt* Vpr induced G2 cell cycle arrest upon caspase 3-inhibition was 12.19% with a standard deviation (SD) of 1.66. In contrast, a change in G2-arrest of −0.32% was observed for the VprP35A virus with an SD of 3.77. The Δvpr virus revealed a change in G2-arrest of −1.62% upon Z-DEVD-fmk treatment with an SD of 0.37.

Taken together and in accordance with previous data (Jacquot et al., 2007), we now demonstrate that NE localization of Vpr is important for G2-arrest induction and that enhanced localization of Vpr at the NE upon caspase 3-inhibition leads to an augmentation of the Vpr-mediated G2-arrest in HIV-1 infected Jurkat T-cells.

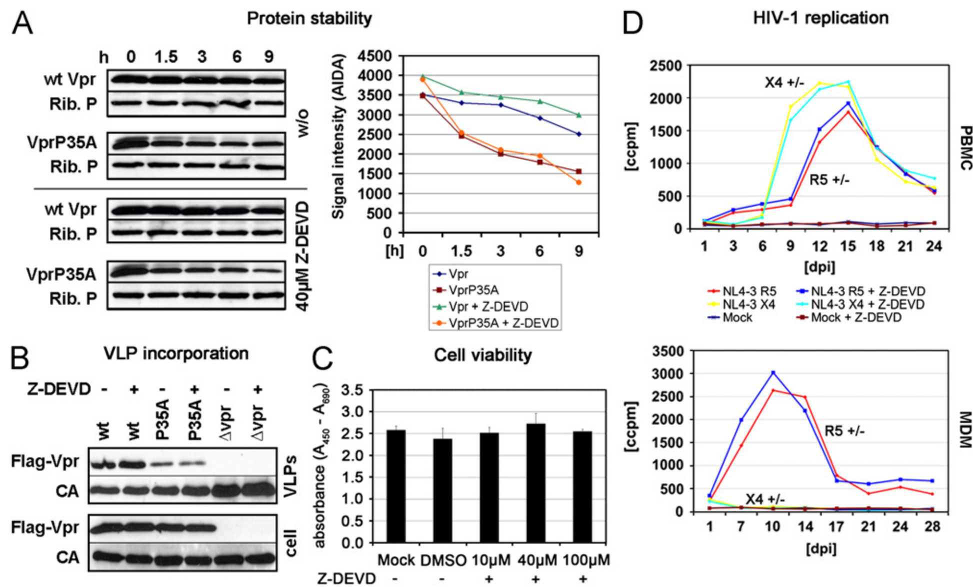

Fig. 3. Effect of Z-DEVD-fmk on functions and properties of Vpr: (A): Left: stability of Vpr under Z-DEVD-fmk treatment. HeLa cells were transiently transfected with FLAG-Vpr expression plasmids. Protein half life was examined by Western blot after blocking protein *de novo* synthesis 24 h post transfection (0 h). Human ribosomal P-Antigen (Rib. P) was used for loading control. Right: densitometric quantification of Vpr-specific bands by image analyzing software (AIDA). (B) Vpr incorporation into HIV-1 virions under caspase 3-inhibition. Virus-like particles (VLPs) were pelleted from supernatants of HeLa cells co-transfected with an *env*- and *vpr*-deficient HIV-1_{NL4-3} sub-clone (Schubert et al., 1999) and FLAG-Vpr expression plasmids containing the indicated mutations. 48 h post transfection VLPs and cell lysates were analyzed for Vpr and CA by specific antibodies in Western blot. (C) Cell viability assay (WST-1) in HeLa cells for Z-DEVD-fmk concentrations up to 100 µM. (D) Replication profiles of X4- and R5-tropic HIV-1_{NL4-3} (NL4-3 R5: sub-clone coding for the 005pt135 V3 region in *env*; (Papkalla et al., 2002)) in PBMCs (upper diagram) and monocyte derived macrophages (MDM; lower diagram), analyzed for viral Reverse Transcriptase activity. R5-tropic strains are depicted in red and blue, X4-tropic strains are shown in light blue and yellow. Uninfected cells are printed in dark blue and brown. Z-DEVD-fmk treatment: red, yellow and brown graph.

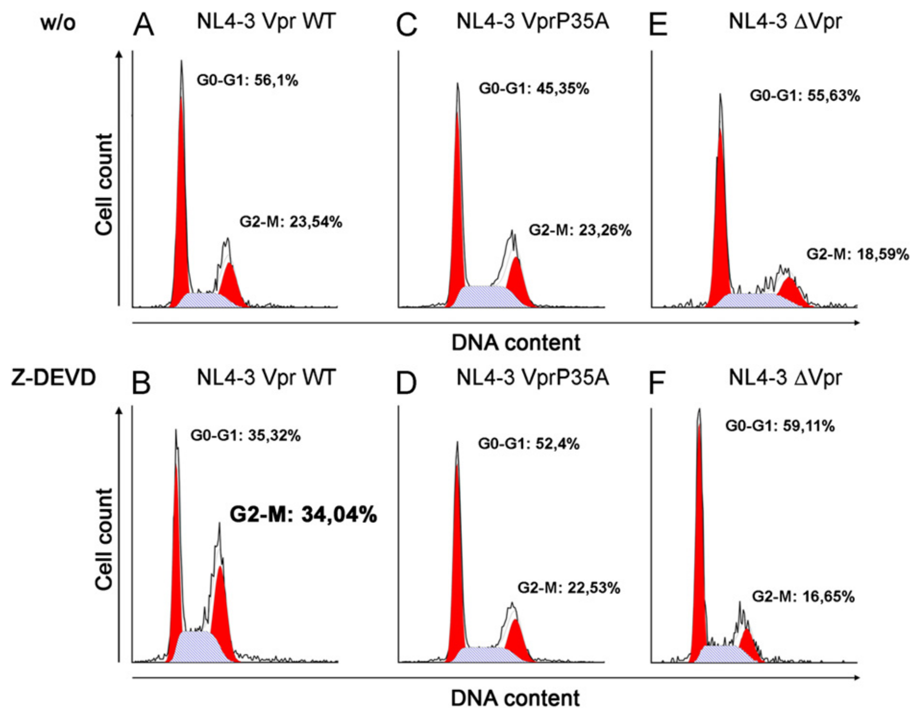

Fig. 4. Effect of caspase 3-inhibition on Vpr-induced G2-arrest: FACS analysis of Jurkat T-cells for DNA content and cell cycle. Cells were infected with HIV-1_{NL4-3} and Vpr mutants thereof and treated with Z-DEVD-fmk 2 h post infection. At peak of replication cells were fixed and permeabilized with 1% PFA and 0.1% Tween20 and stained with anti-p24-FITC and ToPro3-iodite (DNA) for FACS-analysis. (B), (D), (F) Infected Jurkat T-cells, treated with Z-DEVD-fmk. (A), (C), (E) DMSO-treated, infected Jurkat T-cells. Values at G0–G1 and G2-M peaks indicate the percentage of infected cells measured in the indicated cell cycle state.

Because of the difference in the levels of expression and detection of Vpr, localization studies have been performed in HeLa cells transfected with Vpr expression plasmids, while cell cycle was monitored in HIV-1 infected T-cells.

Induction of apoptosis by Vpr is down regulated by caspase 3-inhibition

Previous reports suggest that G2-arrest induction by Vpr might be linked to Vpr's pro-apoptotic activity (Andersen et al.,

2005). Therefore, the apoptosis induction by Vpr was analyzed under caspase 3-inhibition. HeLa cells were transiently transfected with FLAG-Vpr-IRES-eGFP constructs and treated with Z-DEVD-fmk. Cells were then gated for eGFP in flow cytometry and analyzed for Annexin V-staining (Fig. 5A). For both, Vpr- and VprP35A-expressing cells, apoptosis induction was strongly reduced after caspase 3-inhibition. This indicates that Vpr-induced apoptosis requires, at least partially, caspase 3-activity. In consistency, Vpr-induced apoptosis was not completely abrogated even in the presence of 100 μM Z-DEVD-fmk (data not shown). Thus, at least a certain part of the Vpr-induced apoptosis seems to be triggered by caspase 3-independent pathways. Furthermore, the influence of caspase 3-inhibition on Vpr-mediated apoptosis induction seems to be independent of Pro-35, as this mutant is sensitive to Z-DEVD-fmk treatment in terms of cell death other than observed for sub-cellular localization and G2-arrest induction.

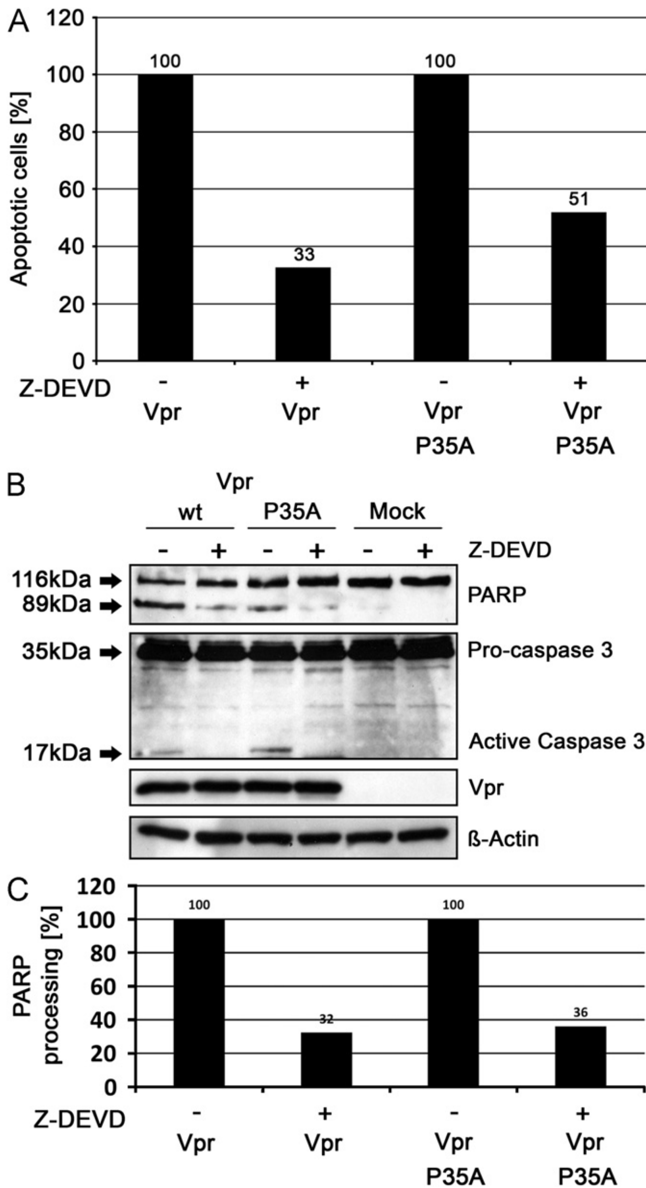

Fig. 5. Effect of caspase 3-inhibition on Vpr-induced apoptosis: (A) HeLa cells were transfected with FLAG-Vpr-IRES-eGFP expression plasmids and treated with 40 μM Z-DEVD-fmk for 24 h. After incubation cells were stained for Annexin V and subjected to FACS analysis. Apoptosis induction was examined by detection of Annexin V signal in eGFP-expressing (Vpr-positive) cells. Apoptosis levels of untreated cells were set to 100%. (B) HeLa cells were transfected with FLAG-Vpr expression plasmids and treated with Z-DEVD-fmk for 24 h. Lanes "Mock" show cells transfected with an empty vector for control. Cell lysates were subjected to Western blot analysis and stained for PARP-1 (116 kDa) and its cleavage products (86 kDa) as well as caspase 3 (35 kDa) and its cleavage products (17 kDa, active caspase 3). For expression control and loading control the blot was stained with anti-Vpr and anti-ß-actin antibody. (C) Densitometric analysis of PARP-signal in Western blot shown in Fig. 5B.

The same result was obtained by Western blot analysis of PARP1-cleavage in pCMV-FLAG-Vpr transfected HeLa cells. The PARP-cleavage is clearly reduced by caspase 3-inhibition (Fig. 5B). In addition, the Western blot was stained with an antibody against caspase 3 (Pro-form) and its cleavage products resulting from activation of caspase 3. Treatment with the caspase 3 inhibitor Z-DEVD-fmk strongly reduced the signal for cleaved caspase 3 as indicated in Fig. 5B.

Although it cannot be completely excluded that apoptosis and G2-arrest induction by Vpr are functionally related to each other, we could show that caspase 3-inhibition causes accumulation of Vpr at the nuclear envelope dependent on the integrity of helix α-1 and that this elevates the Vpr-mediated G2-arrest in HIV-1-infected cells while apoptosis induction was not elevated.

Discussion

The induction of G2 cell cycle arrest is one of the most prominent functions of Vpr and was intensively investigated over the past years (Goh et al., 1998; Hrecka et al., 2007; Lai et al., 2005; Roshal et al., 2003; Zimmerman et al., 2004). Although many details about the underlying mechanism were described, the cellular target that regulates the Vpr-mediated G2-arrest has not been unravelled. G2 arrest induction of Vpr depends on the interaction of Vpr with Cullin 4A via binding to VprBP (DCAF1) and DDB1 (Hrecka et al., 2007). In this complex Vpr most likely acts as a substrate receptor for Cul4A, which in turn induces degradation of a yet unknown cell cycle regulator. It is not fully elucidated in which cellular compartment the complex is catalytically active so far. However, there is evidence that the complex has to be linked to the nuclear envelope (Belzile et al., 2007, 2010; DeHart et al., 2007; Hrecka et al., 2007; Schrofelbauer et al., 2007; Tan et al., 2007) and Cul4A/DDB1/DCAF1/Vpr aggregates have also been observed inside the nucleus (Belzile et al., 2010).

In addition, it was shown that the binding of Vpr to the nuclear envelope *via* interaction with the nucleoporin hCG1 seems to play an important role for G2-arrest induction (Jacquot et al., 2007). Vpr mutants that are no longer able to bind to the NE showed an impaired G2-arrest function (Jacquot et al., 2007). The binding region of Vpr to the NE was mapped to the N-terminal helix α-1 in Vpr. Mutations within the sequence of the Helix (VprL23F and VprK27M) abrogated the binding of Vpr to the NE and showed a strongly reduced arrest of the cell cycle (Jacquot et al., 2007). However, G2-arrest function of Vpr is not solely mapped to the N-terminus of the protein as mutations of certain amino acids in the C-terminus of Vpr (R80 and R90) also lead to an abrogation of G2-arrest function. However, these mutants are still capable of binding to hCG1 (Jacquot et al., 2007).

As it was legitimate to hypothesize that there might be other cellular factors that regulate the binding of Vpr to the nuclear envelope, we analyzed the influence of caspase 3 on Vpr localization, as it regulates nuclear pore morphology during apoptosis and thereby it might influence Vpr localization in cell, particularly since Vpr is capable of shuttling through the nuclear envelope (Bolton and Lenardo, 2007; Depienne et al., 2000; Di Marzio et al., 1995; Lu et al., 1993; Subbramanian et al., 1998; Yao et al., 1995; Zhao et al., 1994). Our data indicate that Vpr's localization at the NE is augmented by caspase 3-inhibition, while the amount of Vpr in the nucleus is reduced. This effect of Z-DEVD-fmk seems to be

specific for Vpr as the localization of another NE-associated protein, RanGAP, is not influenced by caspase 3-inhibition. In addition, no influence of the inhibitor could be observed on the morphology of the nuclear lamina and the allocation of the nuclear pores. These results further indicate that the regulation of the sensitivity of Vpr to caspase 3-activity is related to an intact sequence and structure of helix α-1. As already shown by others, point mutations within the α-1 (e.g., VprL23F) abrogate the binding of Vpr to the NE (Jacquot et al., 2007). Furthermore, this reduced binding could not be recovered by treatment with Z-DEVD-fmk further supporting the role of helix α-1. This was also confirmed by results for the VprP35A mutant, other than VprL23F, that mutant harbours an intact sequence of helix α-1. However, mutation of Pro-35 results in an elongation of the helix towards the C-terminus of the molecule and a significant global change of the structure of Vpr (Votteler et al., 2007). In contrast to VprL23F, the VprP35A mutant is still able to induce G2-arrest (Ardon et al., 2006; Votteler et al., 2007), and most likely binds to the NE. However, the changed structure, caused by the P35A mutation, somehow renders the VprP35A mutant insensitive to caspase 3-inhibition as it does not accumulate at the NE upon Z-DEVD-fmk treatment.

In agreement with the fact that reduced Vpr accumulation at the NE abrogates G2-arrest induction (Jacquot et al., 2007), we could show that augmented accumulation of Vpr at the NE enhances the G2-arrest induction by Vpr in infected Jurkat T-cells.

Due to the limited expression level, detection of Vpr in infected T-cells by confocal microscopy was not possible. Nevertheless, we extrapolate from data in HeLa cells treated with Z-DEVD-fmk that the enhanced G2-arrest induction after caspase 3-inhibition is linked to the accumulation of Vpr at the NE and should also occur in HIV-1 infected cells. This is further in consistency with the fact that VprP35A is insensitive to caspase 3-inhibition in both functions, localization at the NE and G2-arrest induction. In addition, the data for VprP35A indicate that the augmentation of G2-arrest induction is not due to the rescue of infected cells by inhibiting apoptosis. If this was true, G2-arrest induction by VprP35A would have been elevated upon Z-DEVD-fmk treatment.

Furthermore, data for reduced apoptosis induction by both Vpr variants under caspase 3-inhibition indicate that this function most probably is not linked to G2-arrest induction that is elevated by Z-DEVD-fmk treatment.

In addition, we show that apoptosis-induction by Vpr is mainly regulated by caspase 3-activity. However, a certain part of Vpr induced apoptosis seems to be dependent on another cellular pathway due to residual apoptosis induction after Z-DEVD-fmk treatment.

In earlier studies it was postulated that the G2-arrest provides favourable conditions for HIV-1 replication (Brasey et al., 2003; Goh et al., 1998; Hrimech et al., 1999). However, we were not able to show a positive effect of caspase 3-inhibition on the replication of HIV-1. This is most probably due to the moderate increase of G2-arrest induction observed in infected Jurkat T-cells. In addition, since Z-DEVD-fmk first allows to selectively regulate one of the two major functions of Vpr in the context of a *wt* virus, this might indicate that in addition to its capability to induce G2-arrest, other functions of Vpr might be required to support HIV-1 replication in certain cell types. However, it cannot be fully excluded that caspase 3 plays a potential role in the Vpr mediated augmentation of G2-arrest in certain *in vivo* conditions, e.g., in certain cell types, not investigated in this study.

Conclusion

The localization of Vpr at the NE is dependent on the activity of host cell caspase 3 and influences G2-arrest induction by Vpr. In addition, the sensitivity of Vpr to caspase 3 is regulated by helix α-1. The apoptosis induction by Vpr is mainly regulated by the caspase cascade but is not dependent on the N-terminal helix.

Materials and methods

Cell culture and transfection

HeLa cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. For transfection, 5×10^5 cells/well were seeded in 6-well plates 1 d prior to transfection. The next day transfection was performed using Lipofectamine 2000^{TM} (Invitrogen), according to the manufacturer's protocol. Jurkat T-cells were cultivated in RPMI1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Western blot for protein analysis

HeLa cells were transiently transfected with the appropriate DNA. Cells were lysed in cold RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholat, 0.1% Na-SDS, 5 mM EDTA, DNAse, 1 mM PMSF and complete protease inhibitor cocktail (Boehringer Mannheim)). The lysates were cleared by centrifugation at $16000 \times g$ and 4 °C for 10 min. RIPA-soluble proteins and VLPs were separated in 10% SDS/PAA gels, according to Laemmli (1970), transferred onto PVDF membranes (GE Healthcare) and probed with specific antibodies, followed by enhanced chemiluminescence detection. For internal controls, blots were stripped with 0.2% NaOH and re-incubated with appropriate antibodies.

Viruses and virus-like particles (VLPs)

Virus stocks were produced by transient transfection of 293T cells with pNL4-3 plasmids containing the entire HIV-1 genome or a HIV-1_{NL4-3} based sub-genomic expression vector (pNLΔ*env*; (Schubert et al., 1995)) in which *env* was deleted, producing non-infectious VLPs. Virus or VLP containing cell culture supernatant was harvested after 48 h and, after removal of residual cells by centrifugation, passed through a 0.45 µm pore-size filter. Virus was pelleted through 20% (w/v) sucrose ($16000 \times g$, 4 °C, 90 min). Virus stocks were normalized for p24 content as quantified by an enzyme-linked immunosorbent assay (ELISA, Aalto, Dublin, Ireland) and aliquots were stored at −80 °C.

Infection of cells

For infection of Jurkat T-cell cultures, 5×10^6 cells were incubated with 100 ng of p24, and incubated for 12 days, medium and inhibitor were reconditioned every 3rd day. For infection of human PBMCs 1 ng of p24 was applied to 2×10^6 cells in 96-well format, and supernatant was collected every third day post infection. Virus replication was assessed by quantification of the virus-associated RT activity by [^{32}P]-TTP incorporation using an oligo(dT)-poly(A) template as described (Willey et al., 1988).

Flow cytometric analysis

Jurkat T-cells were infected with HIV-1_{NL4-3} or indicated mutants thereof. Parallel cultures were treated with 40 µM Z-DEVD-fmk starting 2 h post infection. Media and inhibitor were reconditioned every 3rd day. 12 d post transfection cells were

harvested, fixed and permeabilized with a solution of 1% paraformaldehyde and 0.1% Tween 20 in PBS for 1 h and stained for viral CA using the KC57-FITC-conjugated antibody (Beckman Coulter). DNA was stained using ToPro3 (Invitrogen). DNA content was then analyzed in infected cells using a FACS LSRII (BD Biosciences) and ModFit software (Verity Software House).

For apoptosis analysis, HeLa cells were transfected with pCMV-FLAG-Vpr-IRES-eGFP plasmids containing the indicated mutations. 24 h post transfection cells were removed from culture dish and stained for Annexin V and Sytox according to manufacturer's protocol (Invitrogen).

Confocal microscopy studies

HeLa cells were transfected with pCMV-FLAG-Vpr expression plasmids or indicated mutants thereof. Parallel cultures were treated with 40 μM Z-DEVD-fmk starting with transfection. 24 h post transfection cells were fixed in 3% paraformaldehyde, permeabilized, using 0.1% Triton X-100 in PBS and stained for FLAG-Vpr using a FLAG-specific FITC-conjugated antibody (Sigma). Images were obtained using a Leica TCS SP5 confocal microscope with a 63 × immersion objective.

The nuclear lamina was stained using the Lamin A/C antibody (Pierce) and the nuclear pore complex was stained using the antibody Mab414 (Abcam). The nucleus was counterstained using DAPI (Invitrogen).

PARP-1 cleavage assay

For PARP-1 cleavage studies cells were transfected with pCMV-FLAG-Vpr constructs as indicated. 24 h post transfection cells were harvested and lysed as described above and subjected to Western blot analysis. PARP-1 and cleavage products thereof were indicated by a PARP-1-specific antibody (Cell Signaling). Caspase 3 and cleavage products thereof were indicated by a caspase 3-specific antibody (Cell Signaling).

Incorporation of Vpr in HIV-1 virions

HeLa cells were transiently co-transfected with the HIV-1 pNLΔ*env*Δ*vpr* (Schubert et al., 1999) and pCMV-FLAG-Vpr constructs. 24 h post-transfection cells were lysed in RIPA-buffer and virions were pelleted from culture supernatant and subjected to immunoblot analysis using anti-FLAG (Sigma) and anti-p24 (Seramun) antibodies.

Determination of protein half life

HeLa cells were transfected with pCMV-FLAG-Vpr plasmids, and 24 h post transfection cells were harvested and transferred in RPMI containing 5 μg/ml cycloheximide, 25 μg/ml emetine and 5 μg/ml puromycin to block protein biosynthesis. Samples were taken after 0, 1.5, 3, 6 and 9 h, and cell lysates were subjected to Western blot analysis. Blots were probed with anti-FLAG and anti-ribosomal P antibodies (Immunovision).

Cell viability assay

For determination of the cell viability under Z-DEVD-fmk treatment 4×10^4 HeLa cells were seeded onto 96-well plates and treated with the inhibitor for 24 h. Cell viability was examined using the cell proliferation reagent WST-1 (Roche) following the protocol of the producer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SS, KF, and MT performed the experimental work. SS, JV, TS, MT and US conceived the experimental strategies and designed individual experiments. SS and KF analyzed the data and SS, JV and US wrote the manuscript. All authors read and approved the final manuscript.

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